

Molecular Cloning and Identification of Novel ω -gliadin Genes from *Triticum* Species

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Gliadin is a main component of gluten proteins that affect functional properties of bread making and contributes to the viscous nature of doughs. In this study, thirteen novel ω -gliadin genes were identified in several *Triticum* species, which encode the ARH-, ATD- and ATN-type proteins. Two novel types of ω -gliadins: ATD- and ATN- have not yet been reported. The lengths of 13 sequences were ranged from 927 to 1269 bp and the deduced mature proteins were varied from 309 to 414 residues. All 13 genes were pseudogenes because of the presence of internal stop codons. The primary structure of these ω -gliadin genes included a signal peptide, a conserved N-terminal domain, a repetitive domain and a conserved C-terminus. In this paper, we first characterize ω -gliadin genes from *T. timopheevi* ssp. *timopheevi* and *T. timopheevi* ssp. *araraticum*. The ω -gliadin gene variation and the evolutionary relationship of ω -gliadin family genes were also discussed.

Keywords: ω -gliadin, *Triticum*, cloning, pseudogene

Introduction

Gluten proteins have been extensively studied because of their effect on the functional properties of dough (Payne 1987; Shewry et al. 1992). These proteins are mainly composed of glutenin and gliadin (Lawrence and Shepherd 1980). Gliadins are monomeric with intramolecular disulphide bonds and contribute to the viscous nature of doughs. The gliadins are subdivided into α -, β -, γ - and ω -types based on their electrophoretic mobility through acidic polyacrylamide gels (Shewry et al. 2003). The ω -gliadin genes are encoded at the *Gli-1* loci on the short arms of chromosomes of homoeologous group 1 which are tightly linked to *Glu-3* loci that encode LMW-GS (Tatham and Shewry 1995). It is estimated that there are 15 to 18 ω -gliadin-related genes per haploid genome: 4–9 on chromosome 1A, 2–5 on chromosome 1B and 2–10 on chromosome 1D (Sabelli and Shewry 1991). The ω -gliadins represent components with a broad *Mr* range of 44–74 kDa (DuPont et al. 2000).

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The ω -gliadins have been classified into four groups, ARE-, ARQ-, KEL- and SRL-, on the basis of their first three amino acids (Kasarda et al. 1983). However, evidence suggests that the KEL-type arises from the ARQ type by the RPGK action of an asparaginyl endoprotease (DuPont et al. 2004). Genes for the first three types are found on the 1A and 1D chromosomes and the encoded proteins are referred to as ω -1 and ω -2 gliadins, respectively. The SRL-type genes are found on the 1B chromosomes and the encoded proteins are referred to as ω -5 gliadins (DuPont et al. 2004). TRQ-type proteins have also been reported in *Triticum monococcum* (Tatham and Shewry 1995) and *Ae. tauschii* (Gianibelli et al. 2002).

The general protein structure of a ω -gliadin is simple: a 19-residue putative signal peptide followed by a 10–11-residue non-repetitive N-terminus, a repetitive region encompassing 90–96% of the protein and a 10–11 residue C-terminus (Hsia and Anderson 2001). The ω -gliadins are composed almost entirely of a repetitive domain bracketed by short unique sequences generally lacking both cysteine and methionine, and are therefore ‘sulfur-poor’ polypeptides (Anderson et al. 2009). Previously reported study suggested that lack of cysteines prevents polypeptides from forming the gluten polymer (Wieser 2007). Up to now, only a few ω -gliadin gene sequences have been reported (Hsia and Anderson 2001; Masoudi-Nejad et al. 2002; Matsuo et al. 2005; Hassani et al. 2008; Chen et al. 2011; Zhuang et al. 2012; Waga and Skoczowski 2014). This is probably because a large repetitive domain in the coding sequence hampers the cloning of ω -gliadin gene (Anderson et al. 2009). Further analysis of the ω -gliadins at the DNA level would provide more information to define the evolution and function of this gene family (Hassani et al. 2008).

In the present paper, we describe 13 novel ω -gliadin genes from *T. urartu*, *T. monococcum*, *T. turgidum* ssp. *dicoccon*, *T. timopheevi* ssp. *timopheevi*, *T. timopheevi* ssp. *araraticum*, and *Ae. tauschii* Cosson. These results would provide with useful information of ω -gliadin gene variation for discussion of the evolutionary relationship of ω -gliadin family genes.

Materials and Methods

Plant materials

Eight accessions were used in this study for molecular cloning of novel ω -gliadin genes, three accessions of *T. urartu* (PI423328, PI428257, CIt17664) and one accession of each species or subspecies: *T. monococcum* ssp. *monococcum* (PI355521), *T. turgidum* ssp. *dicoccon* (PI352365), *T. timopheevi* ssp. *timopheevi* (PI221421), *T. timopheevi* var. *araraticum* (PI352265) and *Ae. tauschii* Cosson (AS60). The accession *Ae. tauschii* Cosson was from Triticeae germplasm collection in Triticeae Research Institute of Sichuan Agricultural University, the remainders were kindly provided by USDA-ARS germplasm bank (<http://www.ars-grin.gov>).

DNA extraction and cloning of ω -gliadin genes

DNA isolation was carried out from young leaf tissue using the Plant Genomic DNA Kit (TIANGEN, Beijing, China). Two pairs of primers were designed: F1 (5'-CTGCAAA-GATGAATGAAACCAC-3') and R1 (5'-ATTTGTCCTGGTTGCTAGGAA-3'), F2 (5'-GGAAAAGCGGCTGCAAAGA-3') and R2 (5'-CAACGATGATCCACTGGT-3'), according to Li et al. (2010) and Chen and Xia (2005) and used to amplify the coding region. PCR amplification was performed using a high fidelity DNA polymerase LA *Taq* polymerase (TaKaRa, Dalian, China) in GC buffer for GC-rich templates. The reaction volume of 50 μ l containing 5 μ l of 10 \times LA PCR buffer, 0.2 mM of dNTPs, 1 μ M of each primer and 2.5 U of LA *Taq* polymerase. The PCR program consisted of an incubation of 95 $^{\circ}$ C for 5 min, followed by 28 cycles of 94 $^{\circ}$ C for 40 s, 58 $^{\circ}$ C for 1 min and 72 $^{\circ}$ for 2 min, and a final extension step at 72 $^{\circ}$ C for 10 min. The PCR products were separated on 1.5% agarose gels and the expected amplicons were recovered, purified and ligated into the pMD19-T vector (TaKaRa, Dalian, China). The ligated mixtures were transformed into *E. coli* DH10B competent cells. The DNA sequencing was performed by Sangon Biotechnology Company (Shanghai, China). At least three independent clones were sequenced to exclude errors.

Sequence alignments and phylogenetic analyses

The prediction of nucleotide sequences was performed using the DNAMAN software (Version 6.0.3, Lynnon Biosoft, Canada). Multiple sequence alignments of nucleotide and the deduced amino acid sequences were completed by Clustal X 2.0 (Larkin et al. 2007). Sequence similarity analysis was performed using the National Center for Biotechnology information (NCBI) standard nucleotide BLAST program against the GenBank nucleotide database (<http://www.ncbi.nlm.nih.gov/BLAST>). The phylogenetic trees were constructed by the Molecular Evolutionary Genetics Analysis software MEGA 6.0 with the substitute model of maximum composite likelihood. In the neighbor-joining (NJ) trees analysis, the gaps were treated as missing data and the boot-strap analysis was conducted with 1000 replicates (Tamura et al. 2013).

Results

Molecular characterization of ω -gliadin genes

The genomic DNA was used as template for PCR amplification with 2 pairs of primers and amplified products were separated on 1.5% agarose gels. The fragments around 1.4 kb (Fig. 1A) and 1.2 kb (Fig. 1B), containing non-coding region, were amplified by the F1R1 and F2R2 primer combinations, respectively. Clones with correct insert size were confirmed by terminal sequencing of previously identified ω -gliadin genes. Thirteen distinct sequences of ω -gliadin genes were identified from more than 50 clones sequenced (Table 1). Out of these 13 distinct sequences, 7 were from C1tr17664, PI423328, PI352265, PI428257, PI355521, AS60 and PI221421, identified by R1F1 primer combination and 6

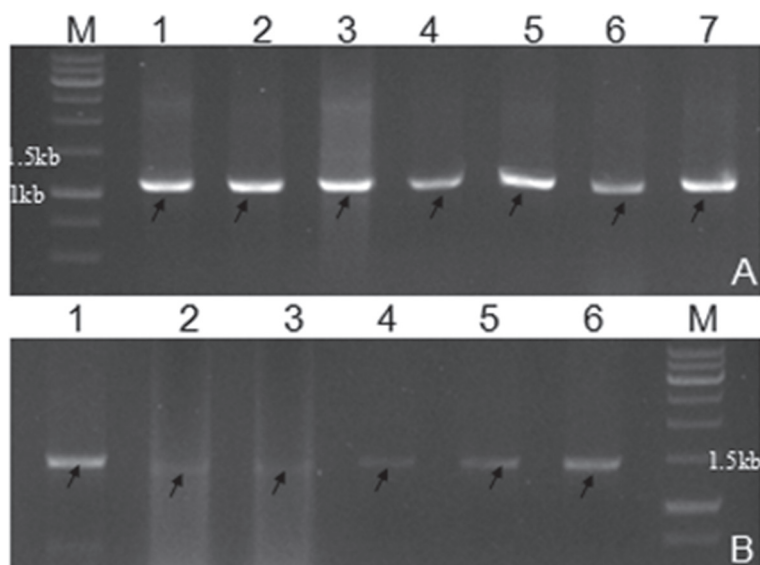


Figure 1. PCR amplified products separated on 1.5% agarose gel. M, DNA Marker DL1000; A) PCR products amplified with primers F1/R1, lanes 1–7 for PI352265, AS60, PI221421, Cltr17664, PI423328, PI428257, PI355521; B) PCR products amplified with primers F2/R2, lanes 1–6 for PI352265, PI352365, AS60, PI221421, Cltr17664, PI428257

were from Cltr17664, PI352265, PI352365, PI423328, AS60 and PI221421, identified by R2F2 primer combination, which were designated as 267 ω -1, 270 ω -1, 272 ω -1, 337 ω -1, 557 ω -1, AS60 ω -1, T ω -1, 267 ω -2, 272 ω -2, 273 ω -2, 337 ω -2, AS60 ω -2, T ω -2, respectively. All 13 genes were pseudogenes because of the presence of internal stop codons (Table 1). BLASTn and p searches confirmed that they were ω -gliadin genes, with sequence lengths varied from 927 to 1269 bp and that these genes could deduce mature proteins from 309 to 414 residues and the deduced molecular weight would range from 35.4 to 48.5 kDa (Table 1). These sequences of the novel ω -gliadin genes were deposited in GenBank with the accession number from KP973445 to KP973457 for 267 ω -1, 270 ω -1, 272 ω -1, 337 ω -1, 557 ω -1, AS60 ω -1, T ω -1, 267 ω -2, 272 ω -2, 273 ω -2, 337 ω -2, AS60 ω -2, and T ω -2, respectively. The sequences alignments are shown in Fig. S1*. In comparison with other genes, there was a 24-bp deletion at the position from 484 to 507 nucleotide (nt), encoding QPQQPFSL in AS60 ω -1, T ω -1, 273 ω -2, AS60 ω -2, and T ω -2. All sequences amplified by primer combination R2F2 are about 275 bp longer than those amplified by R1F1. Sequence alignments indicated that their identity was 84% and that the size of sequences varied mainly due to the In/Del and/or SNPs in the nucleotide sequences. These variations would be attributed to differences in amino acid sequences.

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

Table 1. Comparative analysis of the 13 ω -gliadin genes recovered in this study

Genebank accession	Species	Accession	ω -Gliadin gene	Fragment length (bp)	Number of deduced amino acid	Number of Cys/Met	Q:P:F ration	Deduced molecular weight (kDa)	Similar publised sequence (identity, %)
KP973445	<i>T. urartu</i>	Cltr17664	267 ω -1	951	312	0/0	4:3:1	36.7	KP280196(99)
KP973446	<i>T. urartu</i>	PI423328	270 ω -1	945	307	0/0	4:3:1	36	KF412594(99)
KP973447	<i>T. timopheevi</i> var. <i>araraticum</i>	PI352265	272 ω -1	954	312	0/0	4:3:1	36.7	GQ423431(98)
KP973448	<i>T. urartu</i>	PI428257	337 ω -1	951	310	0/0	4:3:1	36.4	KP280196(99)
KP973449	<i>T. monoccum</i> ssp. <i>monoccum</i>	PI355521	557 ω -1	948	309	0/0	4:3:1	36.3	KF412594(99)
KP973450	<i>Ae. tauschii</i> Cosson	AS60	AS60 ω -1	927	304	0/0	4:3:1	35.7	GQ423431(95)
KP973451	<i>T. timopheevi</i>	PI221421	T ω -1	927	304	0/0	4:3:1	35.4	GQ423431(95)
KP973452	<i>T. urartu</i>	Cltr17664	267 ω -2	1266	413	0/1	4:3:1	48.2	KP280196(99)
KP973453	<i>T. timopheevi</i> var. <i>araraticum</i>	PI352265	272 ω -2	1269	414	0/2	4:3:1	48.5	KP280196(98)
KP973454	<i>T. turgidum</i> ssp. <i>dicoccon</i>	PI352365	273 ω -2	1242	406	0/1	4:3:1	47.4	AB059812(96)
KP973455	<i>T. urartu</i>	PI423328	337 ω -2	1266	413	0/1	4:3:1	48.3	KP280196(99)
KP973456	<i>Ae. tauschii</i> Cosson	AS60	AS60 ω -2	1242	406	0/1	4:3:1	47.4	AB059812(96)
KP973457	<i>T. timopheevi</i>	PI221421	T ω -2	1242	406	0/1	4:3:1	47.4	AB059812(96)

The primary structures of ω -gliadin genes

Thirteen ω -gliadin genes have been cloned and analyzed using ORF Finder and none introns were found in the coding region. It was speculated that these genes were pseudogenes because of the presence of premature termination codons. All the 13 genes were silent with the presence of the internal stop codons (Table 2). The deduced amino acid sequences of these 13 ω -gliadin genes showed that their structures were highly conserved and that they possessed similar primary structure to previously published ω -gliadin genes at the *Gli-1* locus, including a signal peptide with 19 aa, a conserved N-terminal domain with 12 aa, followed by a repetitive domain consisting 90–96% of the protein, and a C-terminus with 10 or 11 aa (Table 2, Fig. S2). Their repetitive domains were rich in glutamine, proline and phenylalanine residues as previously published results. In addition, these ω -gliadin genes were poor in cysteine and methionine residues except for 272 ω -1 with two methionine residues at the repetitive domain and N-terminus, respectively (Table 1).

The different types of ω -gliadin genes

It was found that some of 13 ω -gliadins were different from those previously published according to the classification method of Kasarda et al. (1983). Out of thirteen ω -gliadins, eight belonged to ARH-type, four belonged to ATN-type and one belonged to ATD-type based on their first three peptide residues present in the mature protein (Table 2, Fig. 2). the ATN- and ATD-type have not been reported. Compared to other ω -gliadin genes, the differences in the first three peptide residues present in the mature protein were the sub-

Table 2. The primary structures of ω -gliadin genes we have cloned

Genebank accession	ω -Gliadin gene	Type	Number of amino acid residues				Number of stop codons
			NT	CR	CT	Total	
KP973445	267 ω -1	ARH	12	285	10	312	5
KP973446	270 ω -1	ARH	12	278	9	307	8
KP973447	272 ω -1	ARH	12	284	10	312	6
KP973448	337 ω -1	ARH	12	281	10	310	7
KP973449	557 ω -1	ARH	12	281	9	309	7
KP973452	267 ω -2	ARH	12	382	11	413	8
KP973453	272 ω -2	ARH	12	383	11	414	8
KP973455	337 ω -2	ARH	12	382	11	413	8
KP973450	AS60 ω -1	ATN	12	277	10	304	5
KP973451	T ω -1	ATN	12	277	10	304	5
KP973456	AS60 ω -2	ATN	12	375	11	406	8
KP973457	T ω -2	ATN	12	376	11	406	7
KP973454	273 ω -2	ATD	12	376	11	406	7

stitution of R (S) for T and E (Q, L) for H (D, N) at the position of 21–22 amino acids with nucleotide codon changes from AGG to ACG and GAG (CAG, CTG, CAA) to CAT (GAT, AAT), respectively (Fig. 3).

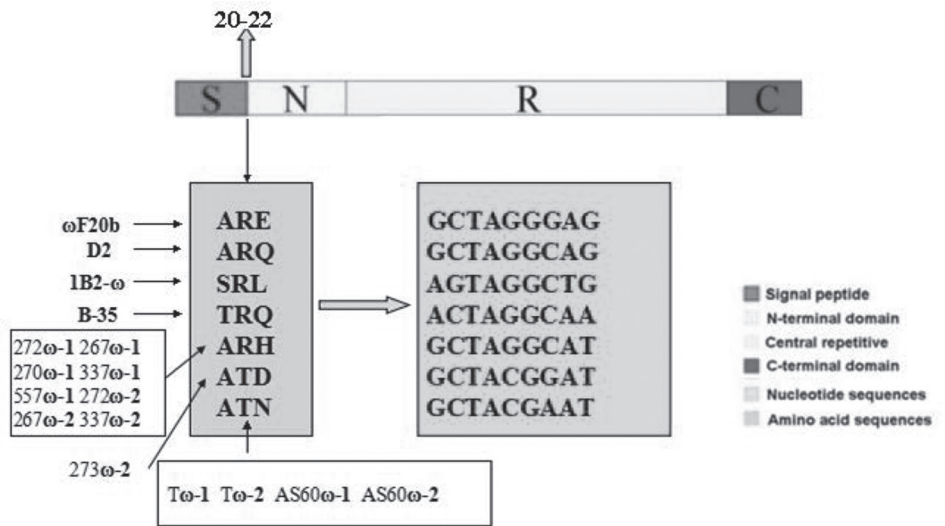


Figure 2. Mutation positions of the different types of ω -gliadin genes cloned in this study

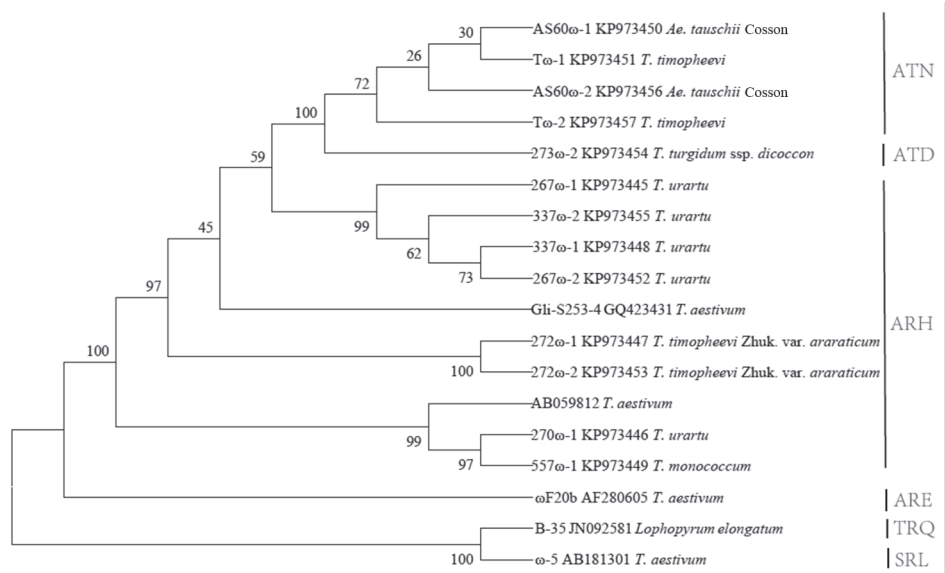


Figure 3. Phylogenetic tree based on the complete amino acid sequences of the 13 ω -gliadin genes cloned in this study and 4 other ω -gliadin genes reported previously

Gene mutation analysis of silence

Base mutation analysis showed that most mutations occurred at the first base of a nucleotide codon, with a frequency of 84%, while mutations at the second base accounted for 16%. Mutation at the third base was not found. The mutations were distributed in the position of 4, 106, 275, 382, 707 and 932 nucleotide. These mutations resulted in substitutions of AAG for TAG, GAA for TAA, TGG for TAG, CAA for TAA, CGA for TGA and CAG for TAG and allowed lysine, glutamine, tryptophane, glutamine, arginine and glutamine codons to change into stop codons (Table S1).

The internal stop codons appeared in 13 different locations in these 13 ω -gliadin genes (Table S1), including one at the signal peptide domain, twelve at the repetitive domain. In addition, all 13 ω -gliadin genes shared the same four of the 14 stop codons at the repetitive domain (Table S1). According to the mutations of amino acid residues, 4 types of ω -gliadin silence genes were identified, which were $K \rightarrow *$, $Q \rightarrow *$, $W \rightarrow *$ and $R \rightarrow *$ (Table S1). Among them, the second type ($Q \rightarrow *$) accounted for 51%, with the highest frequency, while other three types accounted for 49%.

Phylogenetic analysis

To further investigate the evolution of the ω -gliadin genes in this study, a neighbor-joining tree was constructed based on the 13 novel ω -gliadin gene sequences that we cloned and 5 previously reported sequences of ω -gliadin genes that mapped to chromosomes 1A, 1B, and 1D (Fig. 3). This phylogenetic tree demonstrated that 18 sequences were clustered into seven groups, while the 13 novel gliadin genes sequences were grouped into three clades that referred as to the ω -gliadin types: ARH-, ATN-, ATD-, ARE- (ω F20b), TRQ-/SRL- (B-35 and ω -5). Here, ATN- and ATD-types were clustered together into a subclade.

Discussion

Earlier studies demonstrated significant associations between the presence of some gliadin components or alleles and dough strength (Branlard and Dardevet 1985; Wrigley et al. 1982; Wang et al. 2008), and ω -gliadin proteins are one of the most allergenic components of wheat gluten (Waga and Skoczowski 2014). However, few ω -gliadin genes are cloned (Hsia and Anderson 2001; Masoudi-Nejad et al. 2002; Matsuo et al. 2005; Hassani et al. 2008; Chen et al. 2011; Zhuang et al. 2012; Waga and Skoczowski 2014), possibly because their coding sequence includes a large repetitive domain, which hampers gene cloning (Anderson et al. 2009). In this study, we have cloned 13 distinct sequences of ω -gliadins in *T. urartu* (AA), *T. monococcum* ssp. *monococcum* (AA), *T. turgidum* ssp. *dicoccon* (AABB), *T. timopheevi* ssp. *timopheevi* (AAGG), *T. timopheevi* ssp. *araraticum* (AAGG) and *Ae. tauschii* Cosson (DD). The six species have different genomes and the ω -gliadin genes are different. In order to avoid the potential error, some measures to ensure the accuracy of the experiment have been taken, such as multiple independent clones and so on. So, molecular information and correlation analysis of these ω -gliadin genes we generated is reliable and useful.

The ω -gliadins are deficient in both cysteine and methionine, and are therefore “sulphur-poor” polypeptides (Tatham and Shewry 1995). But the cysteine and methionine residues are important (Tatham and Shewry 1995). Out of the 13 ω -gliadin genes, 5 possessed one methionine residue, 1 gene 272 ω -2 ω -gliadin from the *T. timopheevi* ssp. *araraticum* possessed two methionine residues and other 7 genes did not code any methionine residues (Table 1). The representation ratio of Q, P, and F residues in these 13 ω -gliadins is 4:3:1, the same with that of the ARQ/E type (Kasarda et al. 1983; Dupont et al. 2000; Hisa and Anderson 2001). The repeat motif pattern in the 13 ω -gliadin genes is mainly based on PFPQ₀₋₂PQ₁₋₃ (mostly Q is replaced by E, W, L, P, R and some P replaced by S, occasionally F replaced by I) (Fig. S2) which is similar to that of the A- and D-genome ω -gliadin pattern that is based on PFP₁₋₂PQQ (Anderson et al. 2009).

The ω -gliadins have been classified into ARQ/E-, KEL-, SRL-/TRQ-types according to the first three peptide residues present in the mature protein (Kasarda et al. 1983). In this study, we identified two novel types of ω -gliadin genes ATD- and ATN-. Compared to the other ω -gliadin genes, the differences were the substitution of R with T and E (Q, L, H) with D (N) at the position of 21–22 amino acids (Fig. 3). The mechanism of mutation is not clear and needs to be further studied.

Pseudogenes are known to be common in cereal prolamine gene families, such as wheat α -gliadins (Anderson 1991) and ω -gliadins (Hsia and Anderson 2001). ω -Gliadin genes particularly tend to be inactivate when they become large in size (Anderson et al. 2009). In this study, all 13 ω -gliadin genes were pseudogenes with sequences varied from 927 to 1269 bp and they each had its own internal stop codon location. The stop codons randomly distributed on these sequences, respectively. In this study, the changes of amino acids K, Q, W, R to stop codons were resulted from a single base change of A, G or C to T. The average CAA/CAG ratio of the 13 ω -gliadins is 5:1, lower than the reported CAA/CAG ratio of the ω -gliadins 7:1 (Anderson et al. 2009). One consequence of higher frequencies of one codon in homopolymer runs results from increase of unequal recombinations and/or slip-mismatching during replication. These would result in block changes from single repeat unit to large section deletion/duplications of the repetitive domain.

The evolutionary relationship indicated that all the gliadins might have a common evolutionary origin and its evolution should be related to the types of ω -gliadin genes. In the neighbor-joining tree, the ARH- and ATN/D-types of ω -gliadin genes were clustered together but the ATN/D-types were clustered into a subclade, indicating that ATN/D types are divergent from ARH-type. TRQ-/SRL-types ω -gliadin genes were clustered into one clade, which indicated that TRQ-/SRL-types ω -gliadin genes were more closely related. All 13 ω -gliadins are inactive genes and will make no contribution to the wheat seed protein complement but will provide with useful information to explain the evolution of the ω -gliadin gene family of *Triticum* species.

Acknowledgements

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at <http://www.akademai.com/content/120427/>

Electronic Supplementary *Table S1*. Silent mutation analysis of the 13 ω -gliadin genes from *Triticum* species

Electronic Supplementary *Figure S1*. Alignment of the nucleotides sequences of 13 ω -gliadins in this study

Electronic Supplementary *Figure S2*. Alignment of the deduced amino acid sequences of 13 ω -gliadins in this study